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RESEARCH PAPER

Calculated activity of Mn^{2+} at the outer surface of the root cell plasma membrane governs Mn nutrition of cowpea seedlings

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Abstract

Manganese (Mn) is an essential micronutrient for plant growth but is often toxic in acid or waterlogged soils. Using cowpea (*Vigna unguiculata* L. Walp.) grown with 0.05–1500 μM Mn in solution, two short-term (48 h) solution culture experiments examined if the effects of cations (Ca, Mg, Na, Al, or H) on Mn nutrition are related to the root cells' plasma membrane (PM) surface potential, ψ_0^0. When grown in solutions containing levels of Mn that were toxic, both relative root elongation rate (RRER) and root tissue Mn concentration were more closely related to the activity of Mn^{2+} at the outer surface of the PM, $\{Mn^{2+}\}_0^0$ (R^2=0.812 and 0.871) than to its activity in the bulk solution, $\{Mn^{2+}\}_b$ (R^2=0.673 and 0.769). This was also evident at lower levels of Mn (0.05–10 μM) relevant to studies investigating Mn as an essential micronutrient (R^2=0.791 versus 0.590). In addition, changes in the electrical driving force for ion transport across the PM influenced both RRER and the Mn concentration in roots. The $\{Mn^{2+}\}_b$ causing a 50% reduction in root growth was found to be *c*. 500 to >1000 μM (depending upon solution composition), whilst the corresponding value was 3300 μM when related to $\{Mn^{2+}\}_0^0$. Although specific effects such as competition are not precluded, the data emphasize the importance of non-specific electrostatic effects in the Mn nutrition of cowpea seedlings over a 1×10^5-fold range of Mn concentration in solution.

Key words: Manganese nutrition, manganese toxicity, plasma membrane surface potential, root growth, tissue concentration.

Introduction

Manganese (Mn) is an essential micronutrient for the growth of plants, with Mn deficiency often occurring in plants grown on alkaline soils. By contrast, Mn toxicity is an important growth-limiting factor in the acidic soils that comprise *c*. 3.95 billion ha of the global ice-free land or 40% of the world's arable land (Eswaran *et al.*, 1997). Waterlogged soils also often contain high plant-available Mn (Setter *et al.*, 2009). Toxicity of Mn to plants results from excess accumulation in leaves (Wissemeier *et al.*, 1992), but adverse effects of Mn on root growth also occur. Furthermore, uptake of Mn by roots precedes accumulation in the leaves.

It has long been known that variation in the concentrations of cations, such as Ca and Mg, influence the availability of Mn to plants (Vlamis and Williams, 1962; Hauck *et al.*, 2002). Alam *et al.* (2006) reported that increased Ca reduced Mn uptake and, hence, its toxicity in barley (*Hordeum vulgare* L.). The mechanism by which cations reduce the uptake of Mn (and that of other trace metals such as Cu, Ni, or Zn) is not known with certainty, but is commonly

assumed to be due to competition as described, for example, in the biotic ligand model (De Schamphelaere and Janssen, 2002; Paquin *et al.*, 2002). In the context of the present study, this model would relate plant growth to the activity of Mn^{2+} in the bulk solution, $\{Mn^{2+}\}_b$. However, it has been shown that ionic effects on plant growth are often dependent upon the activity of the ion at the outer surface of the root cells' plasma membrane (PM) (see Table 5 of Kinraide and Wang, 2010, for examples). The PM outer surface generally has a negative surface potential, ψ_0^0, increasing the activity of cations and decreasing that of anions in close proximity to it (Kinraide, 2006). Mn-nutritional studies would, therefore, relate plant growth to the activity of Mn^{2+} at the outer surface of the root cells' PM, designated as $\{Mn^{2+}\}_0^0$. The cell wall also has a negative charge, although it appears that this has comparatively little influence on ψ_0^0 and ion concentrations at the PM surface (Gage *et al.*, 1985; Shomer *et al.*, 2003; Kinraide, 2004).

This study examined the effects of cations on the calculated electrical properties of the PM to test the hypothesis that plant growth is more closely related to $\{Mn^{2+}\}_0^0$ that to $\{Mn^{2+}\}_b$. Root growth and Mn concentration in roots were determined in two short-term solution culture experiments using cowpea (*Vigna unguiculata* L. Walp.). Calculated $\{Mn^{2+}\}_b$ and $\{Mn^{2+}\}_0^0$ were based on concentrations of Mn and other cations in solution (Kinraide, 2006). The two experiments separately investigated Mn concentrations considered adequate or excessive for cowpea root growth; the short-term nature of the experiments precluded investigation of the deficient range.

Materials and methods

Experiments were conducted in a laboratory maintained at 25 °C at The University of Queensland, St Lucia, Australia. The experimental system has been used previously (Kopittke *et al.*, 2008, 2009), but is briefly described here. Seeds of cowpea (cv. white Caloona) were rolled in paper towel and moistened with tap water for 3 d. The seedlings were then removed, placed in Perspex strips on top of 600 ml glass beakers filled to the brim (650 ml) with a continuously aerated solution of 1 mM $CaCl_2$ and 5 μM H_3BO_3. After 18 h, the roots were transferred to continuously aerated treatment solutions for a further 48 h. Root length was determined by taking photographs both at the time of transfer (0 h) and after 48 h. Previous investigations using this experimental system have shown that the elongation rate (RER) of roots growing in Ca-sufficient and toxicant-free solutions remain relatively constant during the 48 h experimental period (Kopittke *et al.*, 2009).

Experiment 1 investigated the effects of cations (Ca, Mg, Na, H, and Al) on root growth and on the concentration of Mn in roots at solution concentrations of Mn relevant for toxicity. All solutions contained >2.0 mM $\{Ca\}_0^0$ to prevent a decrease in root growth due to Ca deficiency (Kopittke *et al.*, 2011). Five Mn concentrations were imposed, 1, 250, 650, 1000, and 1500 μM. Because Mn toxicity results from excess accumulation in leaves (Nable and Loneragan, 1984), the Mn concentrations used were higher than those shown to be phytotoxic in longer-term experiments. The five Mn treatments were established in factorial combination with: 1, 7.5, and 20 mM Ca, 1, 5, and 15 mM Mg, 20 mM Na, 0, 2, and 10 μM Al, and two H^+ activities (6.3 and 32 μM, corresponding to pH 5.2 and 4.5). These concentrations of Ca, Mg, Na, Al, and H are known to be non-toxic, reducing the growth of cowpea roots by <10% (Kopittke *et al.*, 2011). Indeed, the ionic strengths (osmolarities) used here are known to be substantially lower than those which reduce the growth of cowpea roots (Kopittke *et al.*, 2011). It is important to note that Al was used as an ameliorant rather than as a toxicant (i.e. Al is added at levels which, although non-toxic, may have substantial impacts upon the effects of toxicants due to changes in ψ_0^0). Therefore, differences in growth between these treatments are not due to toxicities of these cations, but rather due to other effects (such as excess Mn). There were two replicates of the 60 treatments giving a total of 120 experimental units.

Experiment 2 investigated the effects of cations (Ca, Mg, Na, H, and Al, at the same concentrations used in Experiment 1) at five Mn concentrations (0.05, 0.25, 1, 2.5, or 10 μM) considered adequate for root growth in longer term experiments. As for Experiment 1, there was a total of 60 treatments with two replicates.

All solutions contained 5 μM B as H_3BO_3 which must be supplied continuously in the rooting medium (Brown and Shelp, 1997) and Ca at a concentration estimated to result in a minimum of 2.0 mM $\{Ca\}_0^0$ to ensure growth was not limited by Ca deficiency (Kopittke *et al.*, 2011). Chloride salts were used for all treatments. Solution pH was not adjusted in any treatment other than those investigating H^+ or Al for which 0.1 M HCl was used to lower pH where necessary (all solutions with Al added were at pH 4.5). Solution pH was measured at both 0 h and 48 h. The calculated osmolarity of all solutions was <55 mOsM, and all rates of Ca, Mg, Na, H, and Al affected root growth by <10% (Kopittke *et al.*, 2011). Solutions were collected upon completion of the experiment (48 h), acidified using concentrated HCl (32%, 10 M), and refrigerated at 4 °C prior to analysis by inductively coupled plasma mass spectroscopy (ICP-MS) for Mn.

The activities of metal ions in the bulk solution were calculated using PhreeqcI 2.17 (Parkhurst, 2010) with the Minteq database. Final calculations of ψ_0^0 were performed using the Gouy–Chapman–Stern (GCS) model (computer program provided by Dr TB Kinraide, USDA) with the average of the measured pH values in each treatment. The GCS program was modified to allow calculation of activity coefficients using the extended Debye–Hückel equation rather than the Davies equation (Lindsay, 1979). The GCS model combines electrostatic theory (Gouy–Chapman theory) with ion binding (Stern model) so that ψ_0^0 can be computed (Tatulian, 1999; Kinraide and Wang, 2010). This model incorporates the intrinsic surface charge density (σ_0) of a membrane, the ion composition of the bathing medium, and ion binding to the membrane. Knowledge of ψ_0^0 enables the calculation of ion activities at the PM exterior surface. The activity of ion I^Z at the PM exterior surface ($\{I^z\}_0^0$) is computed from the activity of I^Z in the bulk-phase medium ($\{I^z\}_b$) according to the Nernst equation, $\{I^z\}_0^0=\{I^z\}_b\exp[-ZF\psi_0^0/(RT)]$, where Z, F, R, and T are the charge on the ion, the Faraday constant, the gas constant, and temperature, respectively ($F/(RT)=1/25.7$ when ψ_0^0 is expressed in mV).

At harvest (i.e. immediately after photographing the roots after 48 h growth in the treatment solutions), the roots were rinsed briefly in 1 mM $CaCl_2$, blotted dry and weighed, the replicates combined, placed into 50 ml conical flasks, and dried at 65 °C. Thereafter, 10 ml of 5:1 (v/v) nitric:perchloric acid was added. Following digestion, the samples were diluted to 10 ml using deionized water, and the concentration of Mn determined using ICP-MS.

The relative root elongation rate (RRER) and the Mn concentration in roots were analysed statistically using regression analysis, either by linear regression or Weibull equations of the general form

$$RRER = b/\exp\left[(cT)^d\right] \quad (1)$$

where b is the maximum growth rate in toxicant-free and Ca^{2+}-sufficient solutions (b=1 when expressed as RRER), c is a strength coefficient which increases with the strength of the toxicant, T is

toxicant intensity, either $\{Mn^{2+}\}_b$ or $\{Mn^{2+}\}_0^0$ (μM), and d is a shape coefficient (Taylor *et al.*, 1991; Kinraide, 1999). Weibull equations were used where the R^2 value improved substantially compared to the linear regression.

Kinraide (2001) and Wang *et al.* (2011) have suggested that changes in the negativity of ψ_0^0 influence not only the activity of ions at the PM surface, but also the electrical driving force for ion transport across the PM, $E_{m,surf}$, with changes in ψ_0^0 inversely related to changes in $E_{m,surf}$. Whilst the transmembrane electrical potential (E_m) from bulk medium to cell interior can be measured comparatively easily by insertion of microelectrodes into cells (Nobel, 1991) (itself consisting of three potential differences, $E_m=E_{m,surf}+\psi_0^0-\psi_0^i$), the $E_{m,surf}$ is more difficult to determine. Changes in ψ_0^0 have substantial influence on the magnitude of $E_{m,surf}$; given that E_m remains largely constant under typical experimental conditions (Llamas *et al.*, 2000; Kinraide, 2001) any change in ψ_0^0 is offset almost entirely by an inverse change in $E_{m,surf}$. To investigate the possible influence of changes in ψ_0^0 on RRER or the Mn concentration of roots, equation 1 was modified to

$$RRER = b/\exp\left[\left(c_0(1+c_1\psi_0^0)\{Mn^{2+}\}_0^0\right)^d\right] \quad (2)$$

Thus, equation 2 includes the dual effects of ψ_0^0, with changes in the negativity of ψ_0^0 influencing both $\{Mn^{2+}\}_0^0$ and $E_{m,surf}$. In a similar manner, a linear regression ($y=mx+e$) was modified to

$$RRER = m_0\left(1+m_1\psi_0^0\{Mn^{2+}\}_0^0+e\right) \quad (3)$$

where $e=1$ when expressed as RRER. Regression analyses were conducted using SYSTAT 13 (Cranes Software International Ltd). Unless otherwise stated, no coefficients are reported where the 95% confidence interval encompassed zero.

Results

Electrical properties of the plasma membrane

Increased Ca, Mg, Na, H, or Al reduced the calculated negativity of ψ_0^0 (i.e. ψ_0^0 became less negative), decreasing the PM's ability to bind cations. However, the magnitude of the reduction in negativity of ψ_0^0 varied among the cations studied, being a function of ion charge and strength of binding to the PM surface. The predicted effectiveness at reducing the negativity of ψ_0^0 follows the order $Al^{3+}>H^+>Ca^{2+}\approx Mg^{2+}>Na^+$ (Kinraide, 2006; Wang *et al.*, 2008) for cations relevant to the present study. Furthermore, all solutions contained multiple cations (Ca^{2+} and H^+ at a minimum) ensuring that the magnitude of the reduction in ψ_0^0 negativity depended upon (i) the effectiveness of the cation of interest and (ii) the concentrations of other cations present. These two factors, plus small differences in pH, make it difficult to compare treatment effects directly. Nevertheless, theoretical comparisons are possible. For example, a solution at pH 4.5 (32 μM H^+) containing 10 μM Al and 4 mM Ca may be compared with one with 4 mM Ca at the same pH. In this instance, the presence of 10 μM Al would reduce ψ_0^0 by 12 mV. In a similar vein, the reduction in the negativity of ψ_0^0 was calculated as *c.* 5.0 mV for 20 mM Na, 7.4 mV for 32 μM H^+ (cf. pH 5.2), 16 mV for 15 mM Mg, and 32 mV for 20 mM Ca (cf. 1 mM Ca). (Additional comparisons are evident in Supplementary Tables S1 and S2 at *JXB* online for the measured pH of each solution.) These values illustrate the marked effects of Al and H in reducing the negativity of ψ_0^0 relative to those of Ca and Mg and especially that of Na.

It is possible to investigate the influence of changes in ψ_0^0 on $\{Mn^{2+}\}_0^0$ at the same $\{Mn^{2+}\}_b$ using the Nernst equation (Kinraide, 2006). With 1 mM Ca in solution, in which $\psi_0^0=-35$ mV, $\{Mn^{2+}\}_0^0$ would be 15 times higher than $\{Mn^{2+}\}_b$. This is in marked contrast to a solution with 20 mM Ca ($\psi_0^0=-2.9$ mV) in which $\{Mn^{2+}\}_0^0$ would only be 1.3 times higher than $\{Mn^{2+}\}_b$. The addition of 20 mM Na to a solution with 1.5 mM Ca has a much smaller influence on $\{Mn^{2+}\}_0^0$ (see above), with $\{Mn^{2+}\}_0^0$ being 10 times higher than $\{Mn^{2+}\}_b$ at 0 mM Na ($\psi_0^0=-30$ mV) compared with 7.1 times higher at 20 mM Na ($\psi_0^0=-25$ mV).

Root growth

In the absence of Mn stress, there was good root growth during the 48 h experimental period in both experiments (see Supplementary Tables S1 and S2 at *JXB* online). In Experiment 1, the mean (± standard deviation) RER was 1.3±0.1 mm h^{-1} in the 12 treatments which contained a nominal concentration of 1 μM Mn, irrespective of differences in pH and concentrations of Ca, Mg, Na, or Al in solution. Concentrations of Mn in solution considered adequate for plant growth were studied in Experiment 2, and there were no significant effects of Mn on root growth over the range in treatments imposed (RER=1.3±0.1 mm h^{-1}, $n=60$).

A concentration of *c.* ⩾250 μM Mn reduced root growth substantially with 1 mM Ca in solution in Experiment 1 (see Supplementary Table S1 at *JXB* online). Although the addition of up to 20 mM Ca, 15 mM Mg, 20 mM Na, 32 μM H^+, or 10 μM Al in the absence of high Mn reduced growth by <10%, their addition had a marked influence on growth in the Mn-toxic treatments. At pH 5.2, for example, 670 μM $\{Mn^{2+}\}_b$ at 1 mM Ca resulted in a 55% reduction in root growth; 630 μM $\{Mn^{2+}\}_b$ reduced RER by only 18% at 20 mM Ca. Overall, there was a clear distinction between the response in root growth to Mn in solution with 1 mM and 7.5 or 20 mM Ca in solution (Fig. 1A). There was a poor relationship between RRER and $\{Mn^{2+}\}_b$ ($R^2=0.686$) when combining all the data (Table 1). This was in marked contrast to the good relationship ($R^2=0.892$) between RRER and $\{Mn^{2+}\}_0^0$ (Fig. 1B; Table 1), suggesting Mn toxicity is a function Mn^{2+} activity at the outer surface of the PM irrespective of Ca concentration in solution. A 50% reduction in root growth ($EA50_0^0$) of 2900 μM $\{Mn^{2+}\}_0^0$ was calculated.

Evaluated separately, it is evident that additions of cations reduced the adverse effects of high Mn (⩾250 μM) to varying degrees (see Supplementary Table S1 at *JXB* online). A concentration of 1 mM Mg in a solution containing 1 mM Ca alleviated the adverse effects of high Mn by ⩾20% depending on the Mn concentration (230–1500 μM) in the bulk solution. It is difficult, however, to compare the effects of higher Mg concentrations because Ca was also increased to prevent Ca deficiency through low $\{C_a\}_0^0$. Nevertheless, the highest Mg level (15 mM) almost

completely overcame the adverse effect of high Mn on root growth. Decreasing solution pH from 5.2 to 4.5 (i.e. increasing the activity of H^+ from 6.3 μM to 32 μM) similarly alleviated the toxic effects of Mn on root growth by 21–45% depending on the Mn concentration in solution. By contrast, 20 mM Na in solution had, at best, a modest alleviating effect of 12–33%. Interestingly, whilst the addition of 2 μM Al alleviated Mn toxicity, root growth was slightly worse at 10 μM Al.

Combining all the treatments from Experiment 1 provides a more comprehensive comparison of the alleviatory effects of the various cations. As evident with Ca alone (Fig. 1A), there was a comparatively poor relationship (R^2=0.673) between RRER and $\{Mn^{2+}\}_b$ (Fig. 2A). However, the relationship improved with $\{Mn^{2+}\}_0^0$ as the independent variable (R^2=0.812; Fig. 2B). This suggests that the alleviation of Mn toxicity is non-specific and results from changes in $\{Mn^{2+}\}_0^0$ (which is influenced by the negativity of ψ_0^0). Using the appropriate regression (Table 1), the $EA50_0^0$ was calculated as 3300 μM $\{Mn^{2+}\}_0^0$, similar to the value of 2900 $\{Mn^{2+}\}_0^0$ calculated for Ca alone (Fig. 1B).

Besides the importance of $\{Mn^{2+}\}_0^0$, consideration should be given to $E_{m,surf}$ with its influence on ion transport across the PM (Kinraide, 2001; Wang *et al.*, 2011). The incorporation of an additional term besides $\{Mn^{2+}\}_0^0$ to account for the influence of ψ_0^0 on $E_{m,surf}$ (equation 3) improved the relationship further (R^2=0.846; Fig. 2C). The positive value for m_1, 0.0183, suggests that at any given value for $\{Mn^{2+}\}_0^0$, a decrease in the negativity of ψ_0^0 (i.e. an increase in the electrical driving force for ion transport across the PM) decreases root growth (Table 1Table 1; Fig. 2C). This suggests that an increase in the electrical driving force for ion transport across the PM increases the toxic effect of Mn^{2+}.

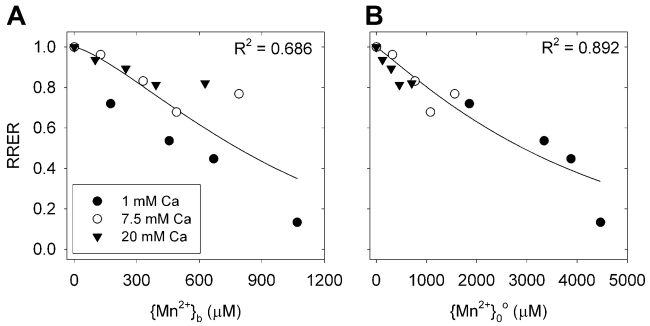

Fig. 1. The effects of Ca in solution on the relationships between the relative root elongation rate (RRER) of cowpea seedlings over 48 h and (A) the activity of Mn^{2+} in the bulk solution, $\{Mn^{2+}\}_b$, and (B) the activity of Mn^{2+} at the outer surface of the root plasma membrane, $\{Mn^{2+}\}_0^0$ (Experiment 1). The legend applies to both panels. Regression coefficients are provided in Table 1.

Mn concentrations in the root

As expected, the concentration of Mn in the root tissue increased with increase in $\{Mn^{2+}\}_b$, but the magnitude was greatly influenced both by the addition of other cations and their concentration (see Supplementary Tables S1 and S2 at *JXB* online). In Experiment 1, for example, the root tissue had a Mn concentration of 400 μg g^{-1} at 670 μM $\{Mn^{2+}\}_b$ with 1 mM Ca, but only 150 μg g^{-1} at 630 μM $\{Mn^{2+}\}_b$ with 20 mM Ca (Fig. 3A). The addition of Mg and H also markedly decreased the Mn concentration in root tissue. The addition of 20 mM Na did not influence the tissue Mn concentration, a finding similar to the effect of Na on root growth. Once again, there was a variable effect of Al on the tissue Mn concentration. In Experiment 1, the addition of up to 10 μM Al tended to increase root tissue Mn concentration, whilst the opposite tendency was evident in Experiment 2.

Table 1. Table 1 Coefficients calculated using SYSTAT for the for the relationships between relative root elongation rate (RRER) or the concentration of Mn (mg kg^{-1}, fresh mass basis) in the root tissue and the activity of Mn^{2+} in the bulk solution, $\{Mn^{2+}\}_b$, activity of Mn^{2+} at the outer surface of the plasma membrane (PM), $\{Mn^{2+}\}_0^0$, and the electrical potential, ψ_0^0, of the outer surface of the PM In no instance did the 95% confidence interval of the coefficients encompass zero. The $\log_{10}$ of $\{Mn^{2+}\}_b$ or $\{Mn^{2+}\}_0^0$ was used as independent variables in Fig. 4D–F. The number of data points (*n*) for the analyses was 15 for Fig. 1 and Fig. 3, and for Fig. 2 and Fig. 4.

Equation	Data source	*b* or *e*	*c* (c_0)	*m* (m_0)	c_1 or m_1	*d*	R^2
$RRER=1/\exp[(c\{Mn^{2+}\}_b)^d]$	Fig. 1A		9.69×10^{-4}			1.34	0.686
$RRER=1/\exp[(c\{Mn^{2+}\}_0^0)^d]$	Fig. 1B		2.47×10^{-4}			1.03	0.892
$RRER=1/\exp[(c\{Mn^{2+}\}_b)^d]$	Fig. 2A		8.17×10^{-4}			1.40	0.673
$RRER=m\{Mn^{2+}\}_0^0+1$	Fig. 2B			-1.57×10^{-4}			0.812
$RRER=m_0(1+m_1\psi_0^0)\{Mn^{2+}\}_0^0+1$	Fig. 2C			-2.47×10^{-4}	0.0183		0.846
$Tissue=m\{Mn^{2+}\}_b+e$	Fig. 3A	–5.41		0.439			0.790
$Tissue=m\{Mn^{2+}\}_0^0+e$	Fig. 3B	27.2		0.101			0.907
$Tissue=m\{Mn^{2+}\}_b+e$	Fig. 4A	0.540		0.356			0.769
$Tissue=m\{Mn^{2+}\}_0^0+e$	Fig. 4B	13.4		0.102			0.871
$Tissue=m_0(1+m_1\psi_0^0)\{Mn^{2+}\}_0^0+e$	Fig. 4C	–0.765		1.73	2.06		0.926
$Tissue=10-b/\exp[(c\log[1000\{Mn^{2+}\}_b])^d]$	Fig. 4D	9.05	0.251			5.13	0.590
$Tissue=10-b/\exp[(c\log[1000\{Mn^{2+}\}_0^0])^d]$	Fig. 4E	9.10	0.209			6.37	0.812
$Tissue=10-b/\exp[(c_0[1+c_1\psi_0^0\log[1000\{Mn^{2+}\}_0^0])^d]$	Fig. 4F	9.02	0.222		0.00163	7.37	0.826

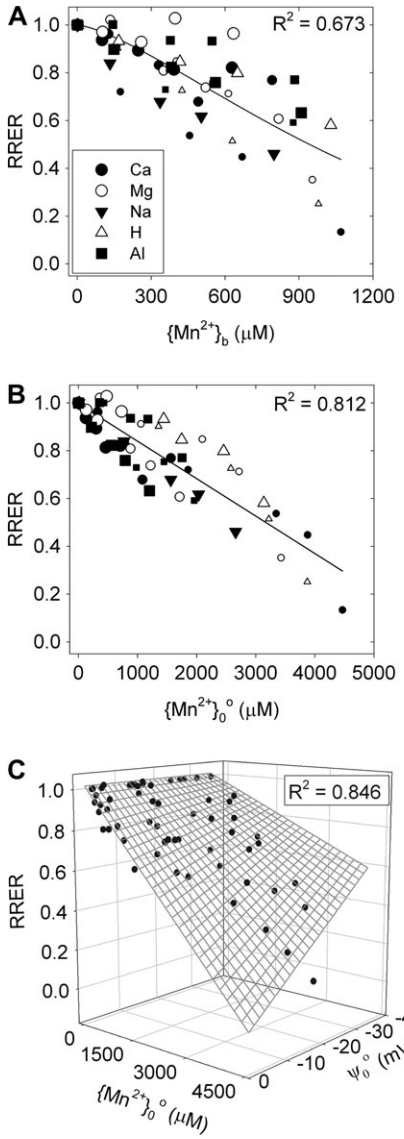

Fig. 2. The relative root elongation rate (RRER) as influenced by (A) the activity of Mn^{2+} in the bulk solution, $\{Mn^{2+}\}_b$, (B) the activity of Mn^{2+} at the outer surface of the root plasma membrane, $\{Mn^{2+}\}_0^0$, and (C) both $\{Mn^{2+}\}_0^0$ and the electrical potential, ψ_0^0, at the outer surface of the plasma membrane (Experiment 1). In (A) and (B), symbol size increases with concentration of each cation. Regression coefficients are provided in Table 1. The legend applies to both (A) and (B).

When the data were combined across all cations, highly significant relationships were obtained between root tissue Mn concentration and $\{Mn^{2+}\}_b$ in Experiment 1 (Fig. 4A) and Experiment 2 (Fig. 4D). As with root growth, the relationship improved with tissue Mn concentration related to $\{Mn^{2+}\}_0^0$ (Fig. 4B, E). Indeed, the R^2 value improved from 0.769 to 0.871 in Experiment 1, and from 0.590 to 0.791 in Experiment 2 (Table 1). These results suggest that the addition of cations influences the availability of Mn due to changes in ψ_0^0 and the consequent effect on $\{Mn^{2+}\}_0^0$. Furthermore, the incorporation of an additional term to account for the influence of ψ_0^0 on $E_{m,surf}$ resulted in improvement, R^2 increasing to 0.926 in Experiment 1 and

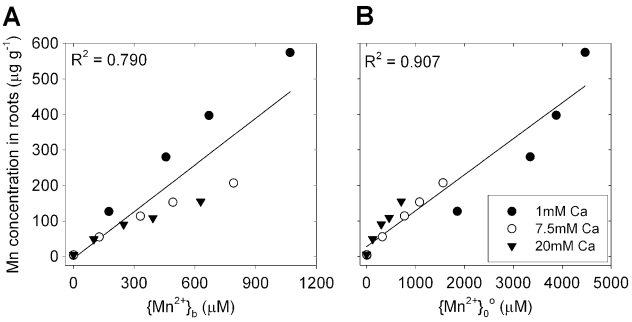

Fig. 3. The effects of Ca on the concentration of Mn in fresh root tissue as influenced by (A) the activity of Mn^{2+} in the bulk solution, $\{Mn^{2+}\}_b$, or (B) the activity of Mn^{2+} at the outer surface of the root plasma membrane, $\{Mn^{2+}\}_0^0$ (Experiment 1). The legend applies to both panels. Information regarding the regression coefficients are provided in Table 1.

0.826 in Experiment 2 (Fig. 4C, F). In both experiments, the values of c_1/m_1 were positive, suggesting that at any given value of $\{Mn^{2+}\}_0^0$, a decrease in the negativity of ψ_0^0 (i.e. an increase in the electrical driving force for ion transport across the PM) increases root tissue concentration of Mn (Table 1; Fig. 4C, F).

Despite the large difference in the nominal Mn concentration in Experiment 1 (1–1500 μM) and Experiment 2 (0.05–10 μM), root tissue Mn was closely and linearly related to $\{Mn^{2+}\}_0^0$ (R^2=0.958, n=120) (combined data plotted on a log–log scale due to the wide range of values). There was further improvement (R^2=0.970) when both $\{Mn^{2+}\}_0^0$ and $E_{m,surf}$ were considered (equation 4).

$$Log\left(1000Tissue\right) = 1000 - 997/\exp\left[\left(0.0160[1 + 0.00244\psi_0^0]\log\left[1000\{Mn^{2+}\}_0^0\right]\right)^{2.57}\right] \quad (4)$$

This allows a good prediction of cowpea root tissue Mn concentration which ranged from 0.71 μg g^{-1} to 570 μg g^{-1} across solutions containing a 1×10^5 range in Mn measured in solution and considerable variation in concentrations of Ca, Mg, Na, or Al and in solution pH which resulted in changes of 0.027–4500 μM $\{Mn^{2+}\}_0^0$ (see Supplementary Fig. S1 at *JXB* online).

Discussion

This study demonstrates the importance of PM electrical properties, and in particular the negativity of ψ_0^0, in influencing plant–Mn interactions in cowpea. For plants grown in solutions containing toxic levels of Mn (Experiment 1), both root elongation and root tissue Mn concentration were more closely related to $\{Mn^{2+}\}_0^0$ than to $\{Mn^{2+}\}_b$ (Figs 2A, B, 4A, B). Similarly, root tissue Mn concentration was more closely related to $\{Mn^{2+}\}_0^0$ than to $\{Mn^{2+}\}_b$ for plants grown in solutions containing Mn at lower levels relevant for plant nutrition (Experiment 2) (Fig.

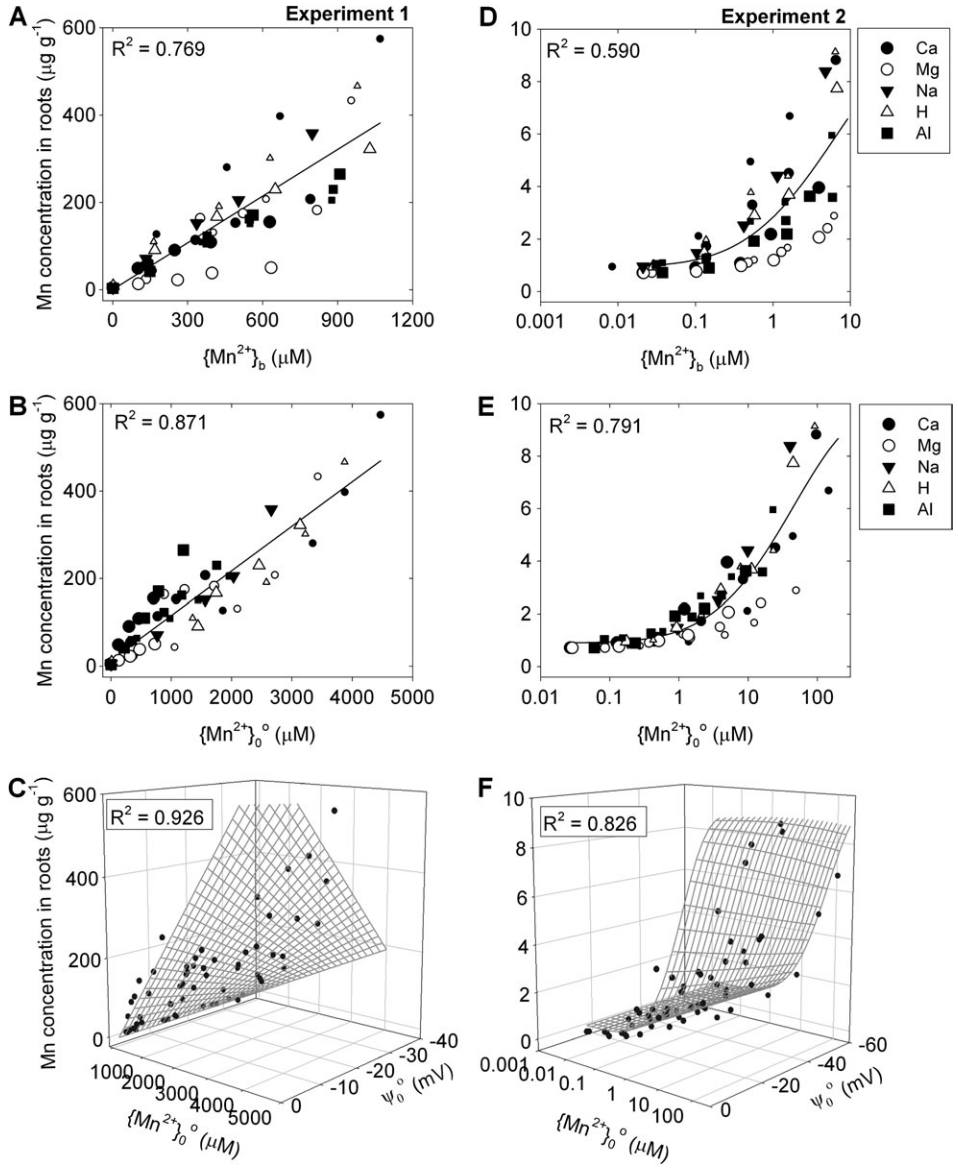

Fig. 4. The concentration of Mn in fresh root tissue in Experiment 1 (left) and Experiment 2 (right) related to (A, D) the activity of Mn^{2+} in the bulk solution, $\{Mn^{2+}\}_b$, (B, E) the activity of Mn^{2+} at the outer surface of the root plasma membrane, $\{Mn^{2+}\}_0^0$, and (C, F) both $\{Mn^{2+}\}_0^0$ and the electrical potential, ψ_0^0, at the outer surface of the plasma membrane. The legends apply to the panels on their left. Symbol size increases with concentration of each cation. Regression coefficients are provided in Table 1. The data for Treatment 65 in Experiment 2 (see Supplementary Table S2 at *JXB* online) were excluded as statistical analyses indicated that this point had high leverage.

4D, E). The data also suggest that ψ_0^0 has a second role in influencing plant–Mn interactions. Specifically, changes in he electrical driving force for ion transport across the PM ($E_{m,surf}$) influence (i) root elongation in Mn-toxic solutions (Fig. 2C) and (ii) tissue Mn concentrations in both Mn-sufficient and Mn-toxic solutions (Fig. 4C, F). In the current study, calculated values of ψ_0^0 have been used as a surrogate for $E_{m,surf}$; a decrease in the negativity of ψ_0^0 increases $E_{m,surf}$ and thus increases the electrical driving force for Mn transport across the PM (Kinraide, 2001; Wang *et al.*, 2011). Overall, this indicates that changes in ψ_0^0 influence plant–Mn interactions through two mechanisms which partially, although not entirely, offset each other. Specifically, the addition of cations decreases the negativity of ψ_0^0 which concomitantly (i) decreases $\{Mn^{2+}\}_0^0$, and (ii) increases the electrical driving force for transport of Mn across the PM ($E_{m,surf}$).

The incorporation of a term accounting for changes in $E_{m,surf}$ along with $\{Mn^{2+}\}_0^0$ improved relationships between root growth and Mn concentration in roots in all three instances (Figs 2C, 4C, F) suggesting that transport across the PM is required for Mn to exert its toxic effect. Compared with other trace metals such as Al and Cu, Mn is readily transported to the shoots (Foy, 1984). This observation regarding the importance of $E_{m,surf}$ for Mn is in contrast to that observed for Cu^{2+} (PM Kopittke, TB Kinraide, P Wang, FPC Blamey, SM Reichman, and NW Menzies, unpublished data). Both Al and Cu bind more

strongly to the cell wall, but further investigation is required to determine if the difference between toxicities of these two trace metals and that of Mn reflects different sites of action.

The results of the current study regarding the importance of ψ_0^0 are emphasized by the re-analysis of published data (Vlamis and Williams, 1962), which demonstrates the importance of $\{Mn^{2+}\}_0^0$ for plant nutrition (see Supplementary Fig. S2 at *JXB* online). In the present study, the magnitude of the effect of the various cations on plant–Mn interactions conform to that which is expected from their effectiveness at reducing the negativity of ψ_0^0 ($Al^{3+}>H^+>Ca^{2+}\approx Mg^{2+}>Na^+$) (Kinraide, 2006; Wang *et al.*, 2008). The addition of comparatively low levels of H^+ (up to 32 μM) or high concentrations of Ca (20 mM) or Mg (15 mM) substantially reduced the negativity of ψ_0^0 thereby reducing Mn uptake and its consequent toxicity; high concentrations of Na (20 mM) had no marked effect. The results regarding Al were unexpected given that the non-toxic concentrations used ($\leqslant$10 μM) decreased the negativity of ψ_0^0 by *c.* 10 mV (see Supplementary Tables S1 and S2 at *JXB* online). This was expected to reduce tissue Mn and its toxicity, but the effects of Al on root growth and tissue Mn concentration was variable (Figs 2A, 4A, D). This is in contrast to that reported for Cu using the same experimental system, in which 10 μM Al consistently reduced the toxicity of Cu (PM Kopittke, TB Kinraide, P Wang, FPC Blamey, SM Reichman, and NW Menzies, unpublished data). The reason for these observations regarding Al is unclear, although it is possible that there is an antagonistic effect between Al and Mn (Taylor *et al.*, 1998). Indeed, Al is highly rhizotoxic, and levels slightly higher than those used in the current experiment are known to reduce root growth.

Although alleviation of Mn toxicity was found to be non-specific in the current study, this does not preclude specific effects in other instances. Several studies have demonstrated that the addition of cations may alleviate toxicities to an extent greater than predicted from electrostatic effects. For instance, Pedler *et al.* (2004) reported that 1–5 μM Mg alleviates Zn toxicity in wheat (*Triticum aestivum* L.), concentrations that are too low to influence the activity of Zn^{2+} at the PM surface. Similarly, Kopittke *et al.* (2011) reported that 200–700 μM K alleviates Na toxicity in cowpea, and Silva *et al.* (2001) reported that 25–50 μM Mg alleviated Al toxicity in soybean (*Glycine max* L. Merr.). In some (but not all) instances, these 'specific' effects occur when the two cations have similar ionic radii. For example, the hydrated ionic radius of Zn^{2+} is 0.430 nm whilst that of Mg^{2+} is 0.428 nm (Volkov *et al.*, 1997).

The $\{Mn^{2+}\}_b$ causing a 50% reduction in root growth ($EA50_b$) in the current study (*c.* 500 to >1000 μM; Fig. 2A) is substantially higher than that found in some other studies. In a meta-analysis of solution culture studies, Kopittke *et al.* (2010) reported that the 25th and 75th percentiles for the toxic concentration of Mn were 5 μM and 180 μM. Analysis of data presented by Edwards and Asher (1982) showed that the Mn concentration causing a 50% reduction for whole plant growth of cowpea was *c.* 4 μM Mn. This apparent discrepancy most likely arises from the short-term nature of the current study (48 h), compared with the average length of 17 d for the studies reported by Kopittke *et al.* (2010) and the 18–31 d of Edwards and Asher (1982). Indeed, for field-grown plants (full life cycle), the toxic effects of Mn are manifest predominantly in the shoots (Nable and Loneragan, 1984), requiring an extended period for Mn accumulation. Regardless, the current study demonstrates the importance of PM electrostatic effects in both the short-term nutrition and toxicity of Mn, with further studies required to examine longer-term effects.

Conclusions

This study demonstrated that short-term root–Mn interactions were related to the electrical properties of the PM in cowpea. For plants grown in solutions containing toxic levels of Mn, both root elongation and root tissue Mn concentration were more closely correlated to $\{Mn^{2+}\}_0^0$ than to $\{Mn^{2+}\}_b$. Similarly, root tissue Mn concentration was more closely related to $\{Mn^{2+}\}_0^0$ than to $\{Mn^{2+}\}_b$ for plants grown at levels of Mn relevant for plant nutrition. Changes in the electrical driving force for ion transport across the PM ($E_{m,surf}$) also influenced both root elongation and Mn concentration in roots. These results demonstrate that, for cowpea, the alleviation of Mn toxicity by cations such as Ca and Mg is non-specific and results from a reduction in the negativity of ψ_0^0. Although the data do not preclude specific effects (such as competition), the data demonstrate the importance of PM electrostatic effects in both the nutrition and toxicity of Mn. Research is needed, however, to establish whether or not the findings have implications for plant Mn status in the longer term.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Table S1. Composition of solutions used in Experiment 1.

Supplementary Table S2. Composition of solutions used in Experiment 2.

Supplementary Fig. S1. Comparison of measured and predicted concentrations of Mn in root tissue for Experiments 1 and 2.

Supplementary Fig. S2. Re-analysis of the data of Vlamis and Williams (1962).

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